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Early Effects of Lasonolide A on Pancreatic Cancer Cells[S]

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ABSTRACT

Lasonolide A, a novel polyketide-derived macrolide, was previously identified from an extract of the marine sponge *Forcepia* sp. in an assay for protein kinase C (PKC) inhibitors. Cytotoxicity testing and profiling of lasonolide A in the National Cancer Institute (NCI) 60 cell panel screen revealed that it was potent toward a broad range of cell lines and also suggested a unique mechanism of action. Contrary to expected results, we found lasonolide A to be a strong activator of PKC in Panc-1 pancreatic carcinoma cells. Downstream mitogen-activated protein kinases, ERK 1/2 and p38 were also rapidly phosphorylated in response to lasonolide A, as was Akt. Microscopy studies revealed that lasonolide A induced blebbing and contraction of the cells within minutes of exposure, and the eventual loss of adherence. However, membrane integrity was maintained and the effects were reversible if lasonolide A was washed from the cells after their loss of adherence. Pretreatment of cells with a myosin II inhibitor, blebbistatin, slowed the early onset, but did not prevent the morphological effects of lasonolide A. Cells stained for actin filaments showed some reduction in stress fiber structure after lasonolide A exposure; however, it did not affect the polymerization of purified actin in vitro. Bisindolemaleimide, a PKC inhibitor, and wortmannin, a phosphoinositide 3-kinase; inhibitor, did not reduce lasonolide A-induced contraction or blebbing or the activation of mitogen-activated protein kinases, although Akt phosphorylation was prevented by wortmannin pretreatment. Our results indicate that lasonolide A activates multiple signal transduction pathways and suggest that the origin is upstream of PKC.

Marine organisms have proven to be a vast source of diverse molecules possessing unique biological activities with high potency. Lasonolide A (Fig. 1), isolated from the Caribbean marine sponge *Forcepia* sp., is no exception. This compound was first isolated by bioassay-guided purification with use of a cell adherence assay to identify novel protein kinase C (PKC) inhibitors and activators (Longley and Harmody, 1991; Horton et al., 1994). In this assay lasonolide A inhibited the phorbol ester-stimulated adherence of EL-4.IL-2 mouse thymoma cells within 30 min with an IC_{50} of 27 nM, but without reducing cell viability during that exposure period (Horton et al., 1994). These results suggested that lasonolide A was a potent inhibitor of PKC. Longer incubation times (48–72 h) revealed that lasonolide A was highly cytotoxic to cultured mammalian cancer cells with TC_{50} values in the range from 3 to 57 nM. Submission of lasonolide A for assay in the United States National Cancer Institute (NCI) *In Vitro* Cell Line Screening Project (Shoemaker, 2006) confirmed its cytotoxicity and suggested a unique mechanism of action when lasonolide A was analyzed by use of the COMPARE algorithm.

Initial cytotoxicity assays on lasonolide A revealed a potent activity toward cells of pancreatic origin. Because pancreatic cancer has a poor prognostic outcome upon diagnosis and is often refractory to current chemotherapies, a further examination of the mechanism of lasonolide A cytotoxicity was warranted. However, efforts to conduct these studies were hampered by the limited availability of the sponge and difficulty in the synthesis of lasonolide A. Given the challenge of its unique chemical structure and interesting biological activity, several chemistry research groups have recently explored feasible synthetic pathways which would provide sufficient lasonolide A for future biological studies (Kang et al., 2003; Yoshimura et al., 2006; Ghosh and Gong, 2008). The studies presented here were conducted by use of natural

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ABBREVIATIONS: BIM-1, bisindolemaleimide; PKC, protein kinase C; MARCKS, myristoylated alanine-rich protein kinase C substrate; ERK1/2, p44 and p42 extracellular signal-regulated protein kinases 1 and 2; FITC, fluorescein isothiocyanate; PMA, phorbol 12-myristate 13-acetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; PI3K, phosphoinositide 3-kinase; DPBS, Dulbecco's phosphate-buffered saline; MAP, mitogen-activated protein.

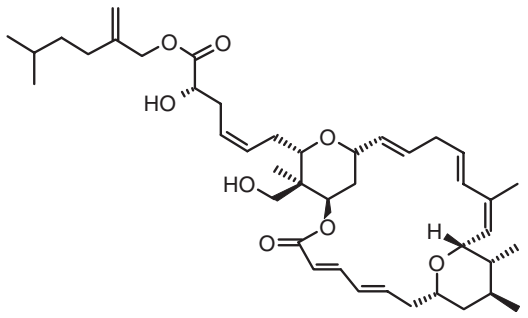

Fig. 1. Lasonolide A chemical structure.

lasonolide A isolated from *Forcepia* sp. collected in the U.S. Gulf of Mexico in 2003 (Wright et al., 2004). As part of a series of on-going studies into the pharmacology of lasonolide A, we report on the unexpected stimulatory effect of this compound on PKC in Panc-1 cells as well as its rapid induction of a reversible blebbing and contraction response.

Materials and Methods

Collection of Sponge Source Material and Isolation of Lasonolide A. Lasonolide A used in these studies was derived from samples of *Forcepia* sp. sponge collected by manned submersible in the U.S. Gulf of Mexico and isolated as described previously (Wright et al., 2004). The chemical structure of lasonolide A and sample purity were confirmed by NMR and HPLC chromatography.

Chemicals and Antibodies. Phosphorylation specific rabbit antibodies to PKCα/βII (Thr638/641), PKC pan (βII Ser660), myristoylated alanine-rich protein kinase C substrate (MARCKS; Ser152/156), p44 and p42 extracellular signal-regulated protein kinases 1 and 2 (ERK1/2; Thr202/Tyr204), p38 MAP kinase (Thr180/Tyr182), Akt (Thr308 and Ser407), a horseradish peroxidase-conjugated anti-rabbit, and molecular weight protein ladder were purchased from Cell Signaling Technologies (Danvers, MA). FITC-phalloidin actin stain and the SlowFade antifade reagent were from Molecular Probes (Eugene, OR). Purified rabbit skeletal muscle actin and bovine brain tubulin were purchased from Cytoskeleton Inc. (Denver, CO). All tissue culture media, serum, and supplements were purchased from Invitrogen (Carlsbad, CA). Rabbit antiactin, blebbistatin, wortmannin, bisindolylmaleimide-1 (BIM-1), phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), verapamil, and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted and were of the highest grade available. Kabiramide C (Matsunaga et al., 1986) was obtained from the Harbor Branch Oceanographic Institute pure compound library.

Cell Culture and Treatment. The human pancreatic carcinoma cell lines Panc-1, AsPC-1, and CFPAC-1, and the human breast adenocarcinoma lines MCF-7 and NCI/ADR-RES (formerly MCF-7/ADR) were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 tissue culture medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 60 mg/ml L-glutamine, 18 mM HEPES, 0.05 mg/ml gentamicin, and 10% fetal bovine serum. The cells were cultured in plastic tissue culture flasks and kept in an incubator at 37°C in humidified air containing 5% CO_2. All cells were subcultured 1:10 to 1:20 every third or fourth day using a 0.5% trypsin, 0.53 mM EDTA solution and used within 20 passages of the initial stock culture.

Western Immunoblotting. Sixteen hours before assay with lasonolide A a subculture set of Panc-1 cells grown to 70% confluence were transferred to culture media with reduced (2%) fetal bovine serum content. In experiments involving inhibitors (BIM-1 or wortmannin), the cells were preincubated with the compound for 30 to 60 min before the addition of lasonolide A. After exposure to test agents, the cells were washed once in cold Dulbecco's phosphate-buffered saline (DPBS) and resuspended in cold radioimmunoprecipitation assay buffer (50 nM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). The cells were incubated on ice in radioimmunoprecipitation assay buffer for 1 h and centrifuged at 15,000*g*, 4°C for 15 min. The supernatant was collected and aliquoted for storage; the protein concentration was determined by use of a biuret reaction method (Pierce Biotechnology, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nylon membranes for probing with the selected antibodies.

Viability and Recovery Assays (MTT and Lactate Dehydrogenase). The antiproliferative effects of lasonolide A were evaluated by a modification of the methods by Alley et al. (1988). In brief, cell populations in log-phase growth were enumerated by measuring the mitochondrial metabolism of MTT after 72-h exposure to serial dilutions of lasonolide A. Positive controls were included to verify the sensitivity of each cell line. These included varying dilutions of 5-fluorouracil and/or doxorubicin. A linear relationship between cell number and MTT metabolism was routinely observed over the range of cell densities used in these experiments. The concentration of the agent causing 50% cytotoxicity (TC_{50}) and the 95% confidence intervals were calculated by nonlinear regression of log-transformed data combined from a minimum of three experiments (Prism; GraphPad Software, San Diego, CA). A minimum of seven concentrations, each comprising 4 to 5 wells, was used in the derivation of the IC_{50}.

The effect of lasonolide A on Panc-1 cell membrane integrity was assessed by measuring the efflux of lactate dehydrogenase (LDH) from live cells. Cells were subcultured into 96-well plates at a density of 2×10^4 cells/well and allowed to adhere overnight at 37°C. The culture medium was removed from all wells and replaced with serum-free media containing lasonolide A. Controls consisted of serum-free culture media only, or cells incubated with serum-free media, 0.5% Triton X-100, and 0.5% Triton X-100 + 500 nM lasonolide A. The plate was incubated at 37°C for 30 or 60 min, centrifuged at 250*g* for 10 min, and 100 μl of the culture media was transferred to a second 96-well plate. LDH activity was determined by the addition of 100 μl of a proprietary test reagent containing diaphorase and 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (Roche Applied Science, Indianapolis, IN) and incubating at room temperature for 30 min before measuring absorbance at 490 nm. Under these test conditions the reduction of 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride to its red formazan salt was found to be proportional to LDH activity.

For wash-out experiments, Panc-1 cells were grown to near confluence in tissue culture flasks. The cells were exposed to 50 nM lasonolide A for 2, 4, or 8 h, the nonadherent cells collected, washed, and centrifuged twice at 400*g* for 10 min, and resuspended in fresh culture media. Cell counts and viability were determined by trypan blue exclusion. An equal number of viable cells were then transferred to 96-well plates and allowed to grow in the absence of lasonolide A for 48 h. Cell recovery was measured against untreated Panc-1 cells that were removed from the initial culture flask by the use of trypsin.

Microscopy. Live pancreatic carcinoma cell lines were observed by differential interference contrast microscopy using a confocal microscope (Olympus America, Mellville, NY) equipped with a thermoelectric heated stage. Cells were subcultured into six-well plates containing sterile glass coverslips, at a density of 7×10^5 cells/well, and allowed to adhere for 1 to 2 days in a 37°C incubator. Coverslips were transferred to a 60-mm Petri dish containing warm culture media and observed by use of a submersible 60× lens. The culture media was carefully aspirated from the Petri dish by syringe and replaced with prewarmed media containing lasonolide A. Digital images were captured every 30 s after the addition of compounds.

To observe the actin cytoskeleton, cells were cultured onto sterile glass coverslips as indicated above. After their exposure to lasonolide

A or other test agents, the cells were rinsed briefly in warm DPBS supplemented with 0.32 M sucrose. Coverslips containing the adhered cells were fixed in 4% formaldehyde in DPBS/sucrose for 10 min at room temperature and then permeabilized in 0.5% Triton X-100 in DPBS/sucrose for 5 min. The coverslips were rinsed twice in DPBS and incubated for 20 min with FITC-phalloidin diluted in DPBS according to the manufacturer's instructions. After several rinses in DPBS the coverslips were air dried and mounted on slides with SlowFade antifade solution (Molecular Probes). Cells were observed by epifluorescent microscopy. The effects of lasonolide A on the microtubule matrix in cultured cells were conducted as described previously (Isbrucker et al., 2003).

Actin and Tubulin Polymerization Assays. The effects of lasonolide A on actin polymerization were tested by the semiquantitative measurement of pelleted actin filaments (Cooper and Pollard, 1982). Purified rabbit skeletal muscle actin was resuspended to 0.4 mg/ml in cold buffer G (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$) and divided into 0.08-mg aliquots. Lasonolide A or control agents were added to each aliquot and the samples incubated on ice for 30 min. Controls included kabiramide C, a known actin-depolymerizing agent (Tanaka et al., 2003), and DPBS. One tenth the volume of polymerization buffer (500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP) was added to all aliquots that were then incubated at room temperature for 1 h. All samples were centrifuged at 100,000*g* for 1 h at 20°C, the supernatants collected, and the pellets resuspended to their original volume in buffer G.

Aliquots from all supernatant and pellet samples were diluted 1:2 with SDS-polyacrylamide gel electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 1.2% SDS, 10% glycerol, 20 mM dithiothreitol, 0.01% bromphenol blue) and denatured by heating to 95°C for 5 min followed by rapid cooling on ice. An equivalent volume of the samples were loaded into each well of 10% Tris gels (Bio-Rad Laboratories, Hercules, CA) and separated by electrophoresis. Gels were removed from the apparatus and frame, washed in three changes of deionized water for 15 to 30 min, and stained with Coomassie blue (Pierce Biotechnology) overnight. On the next day, the gel was destained by several washes in deionized water over a period of 1 to 2 h. The gels were imaged on an Epson Perfection 4870 Photo flatbed optical scanner.

The effect of lasonolide A on tubulin polymerization dynamics were conducted as described previously (Isbrucker et al., 2003). In brief, 10 μM lasonolide A was added to a solution of 1 mg/ml purified bovine brain tubulin in nonpolymerizing buffer (1 mM GTP, 80 mM PIPES, 1 mM EGTA, 0.5 mM magnesium chloride, pH 6.8) and changes in optical density were monitored at 350 nm in a Hitachi spectrophotometer equipped with an electronic thermostatted cell holder. The temperature was held at 4°C for 1 min and then gradually increased to 35°C over a 30-min period. Under these conditions tubulin does not polymerize without an agent that reduces its critical concentration. The marine-derived compound discodermolide was used as a positive control. The effects of 10 μM lasonolide A on preformed microtubules were similarly monitored by promoting the hyperstabilization of 10 mg/ml tubulin in the nonpolymerizing buffer supplemented with 10% glycerol. Under these conditions microtubules spontaneously form at 35°C and disassemble when cooled (6°C).

Results

Cytotoxicity. The cytotoxicity of lasonolide A was tested in Panc-1, MCF-7, and NCI-ADR cells after 72-h exposure. The concentration reducing the Panc-1 cell number by 50% (TC_{50}) was 68 ± 10 nM, whereas the TC_{50} for MCF-7 and NCI-ADR cells was 25 ± 12 and 90 ± 5 nM, respectively. The inclusion of 2 μM verapamil in the cytotoxicity assay did not significantly alter the TC_{50} of lasonolide A to NCI-ADR cells.

Data acquired from the submission of lasonolide A to the 60-cell panel screening program at NCI revealed a wide range of toxicity after 48-h continuous exposure (supplemental data from the National Cancer Institute). TC_{50} values in this panel ranged from approximately 2 nM in the HL-60 human leukemic cell line to above the maximum concentration tested of 1 μM in six of the cell lines tested. Analysis of the data using the COMPARE algorithm did not suggest a comparable mechanism of action.

Signal Transduction. The effect of lasonolide A on PKC was investigated by monitoring the phosphorylation of PKC as well as MARCKS, a substrate of PKC. Lasonolide A (50 nM) induced a small increase in the phosphorylation of PKC after 10-min incubation with a stronger increase at 30 min (Fig. 2A). This effect was similar when measured with a pan-specific phospho-PKC (Ser660) antibody, or with an antibody toward the calcium-dependent PKCα/β_{II} (Thr638/641) isoforms. The phosphorylation of PKC was closely followed by that of MARCKS (Ser152/156), indicating PKC enzyme activation (Fig. 2B). The activation of two MAP kinases that can potentially lay downstream of PKC were also investigated in Panc-1 cells exposed to lasonolide A. Both p38 and ERK1/2 MAP kinases showed increased phosphorylation (Fig. 2B), although maximal level was reached within 10 min and maintained at 30 min. Preincubation of Panc-1 cells with 2 μM BIM-1 for 30 min before lasonolide A exposure greatly inhibited the phosphorylation of MARCKS, but had no effect on the phosphorylation of either MAP kinase (Fig. 2B).

To help identify the origin of PKC activation by lasonolide A, wortmannin was used to evaluate the potential upstream involvement of a phosphoinositide 3-kinase (PI3K) pathway. Although PDK1 would ideally be monitored as an intermediary between PI3K and PKC, it was found to be ubiquitously phosphorylated in our hands regardless of cellular quiescence or activation (results not shown). However, phosphorylation of Akt at threonine 308 was increased in response to

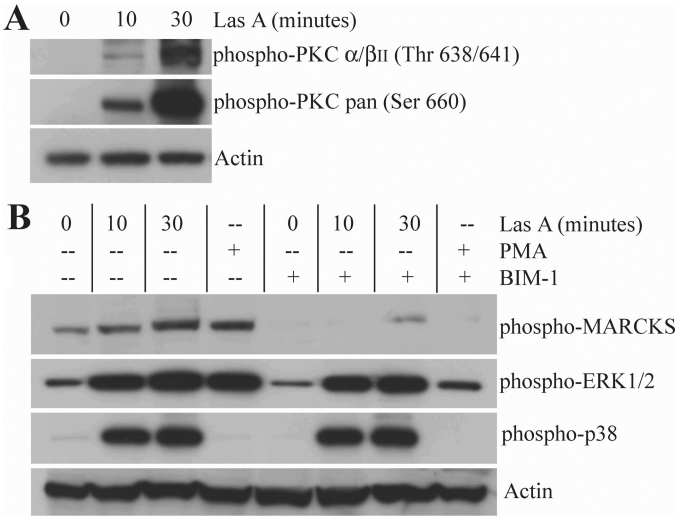

Fig. 2. A, incubation of Panc-1 cells with 50 nM lasonolide A for 10 and 30 min induces the phosphorylation of PKC. B, lasonolide A also induced the phosphorylation of MARCKS (Ser152/156), ERK1/2 (Thr202/Tyr204), and p38 (Thr180/Tyr182) kinases. A 30-min preincubation of Panc-1 cells with 50 μM BIM-1 before the addition of lasonolide A abrogated the phosphorylation of MARCKS by lasonolide A and the positive control, PMA. BIM-1 pretreatment did not reduce the effects of lasonolide A on the phosphorylation of either ERK1/2 or p38. Actin was monitored to verify the equal loading of total proteins per lane. Each experiment was conducted three times with similar results.

lasonolide A (Fig. 3A), indicating the activation by PDK1. Serine 473 of Akt was similarly phosphorylated in response to lasonolide A exposure. The preincubation of Panc-1 cells with 500 nM wortmannin for 30 min before the addition of lasonolide A inhibited threonine 308 phosphorylation of Akt, indicating the involvement of PI3K in lasonolide A signaling. However, neither PKC, ERK1/2, nor p38 kinase phosphorylation was reduced by the pre-exposure of cells to wortmannin (Fig. 3B).

Lasonolide A Effects on Cell Morphology and Cytoskeletal Matrix. While studying the signal transduction effects of lasonolide A, we observed that exposed cells rapidly lost their adherence to the culture dish. Further observation by contrast light microscopy of Panc-1 cells exposed to 50 nM lasonolide A revealed a contraction of the adherent cells from the culture surface accompanied by blebbing of the membrane (Figs. 4, A–D). These effects did not commence uniformly throughout the culture and were observed in some cells as early as 3 min after the addition of lasonolide A, with the remaining cells following suit within 60-min exposure. All cells lost their attachment to the culture surface in 20 to 30 min after the onset of the blebbing process. The cell nucleus remained intact and without notable morphological change during the blebbing and contraction events. Similar morphological changes were also observed in AsPC-1 (Supplemental Fig. 1) and CFPAC-1 (Supplemental Fig. 2) human pancreatic carcinoma cells indicating that the response to lasonolide A is not unique to the Panc-1 cell line. Preincubation of Panc-1 cells with 2 μM BIM-1 or 500 nM wortmannin for 30 min did not reduce the morphological changes caused by the addition of 50 nM lasonolide A.

To verify the role of the actin/myosin complex in the morphological effects of lasonolide A, Panc-1 cells were preincubated for 30 min with 50 μM blebbistatin, an inhibitor of myosin II activity. Blebbistatin alone had no discernible effects on Panc-1 cell morphology but delayed the onset and extent of the early contraction and blebbing effects of 50 nM lasonolide A (Fig. 4, E and F). However, the inhibitory effects of blebbistatin were limited as the morphological changes within 30 to 60 min of the addition of lasonolide A were indistinguishable from cultures not exposed to blebbistatin.

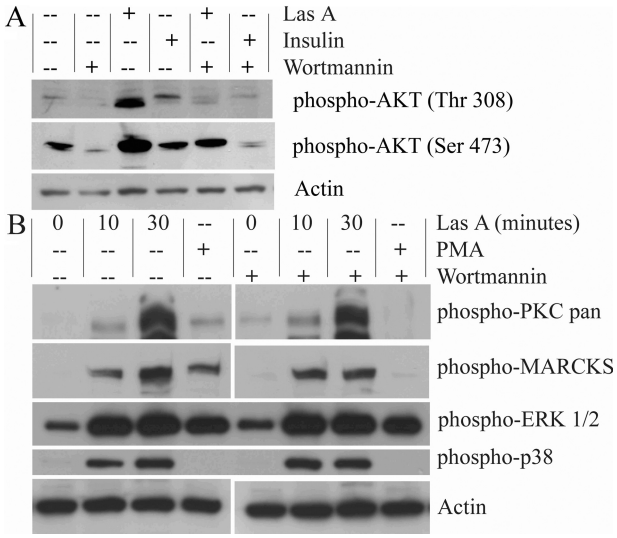

Fig. 3. A, lasonolide A and insulin both promote the phosphorylation of Akt at Thr308 and Ser473. This effect on phosphorylation at Thr308 is abolished by 1-h pretreatment of Panc-1 cells with 500 nM wortmannin before the addition of either stimulating agent. B, wortmannin did not inhibit lasonolide A-induced activation of PKC, or of MARCKS, ERK1/2, or p38. The activity of wortmannin was confirmed by the reduction in PKC and MARCKS phosphorylation induced by PMA. Actin was monitored to verify the equal loading of total proteins per lane. Experiments were conducted three times with similar results.

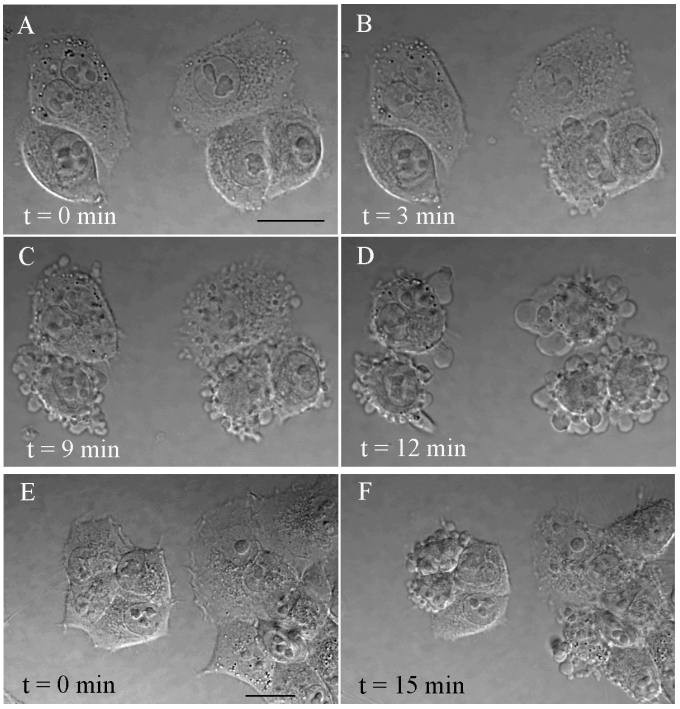

Fig. 4. Panc-1 cells were grown on glass coverslips and observed by time-lapse differential interference contrast microscopy. Cells were incubated with 50 nM lasonolide A for 0 (A), 3 (B), 9 (C), or 12 (D) min, and show a contraction and blebbing of the membrane. Preincubation of cells with 50 μM blebbistatin for 30 min (E) show no changes in cellular morphology. This pretreatment reduced the initial contraction and blebbing response induced by the addition 50 nM lasonolide A for 12 min (F), but these inhibitory effects were no longer apparent within 30 to 60 min of the addition of lasonolide A. Bars (A, E) = 20 μm. These experiments were conducted a minimum of three times.

Staining of untreated Panc-1 cells with FITC-phalloidin revealed an evenly distributed, fine network of actin stress fibers, microfilaments, and filopodia (Fig. 5A). The effects of lasonolide A on the actin cytoskeletal matrix were not uniform throughout any single preparation, which is possibly a reflection of the staggered response time of cells to this compound. Although many cells that had not started the retraction process retained their microfilaments and stress fibers, some cells (Fig. 5B, arrow heads) had a reduced or absent actin matrix not observed in untreated cultures. Cells undergoing contraction frequently showed exaggerated filopodial protrusions (Fig. 5B, asterisk) which remained with the cell as it lost adherence (Fig. 5C). Although some bleb structures induced by lasonolide A stained strongly for actin (Fig. 5B, arrow), the majority had a very limited actin content (Fig. 5C, arrow). Lasonolide A had no effect on the microtubule matrix in cultured cells.

Actin and Tubulin Polymerization. A spin-down assay was used to determine whether lasonolide A inhibited the polymerization of purified actin in vitro. Rabbit skeletal muscle actin was incubated in polymerization buffer in the presence or absence of lasonolide A. Kabiramide C, an actin-depolymerizing agent, and a nonpolymerizing buffer were

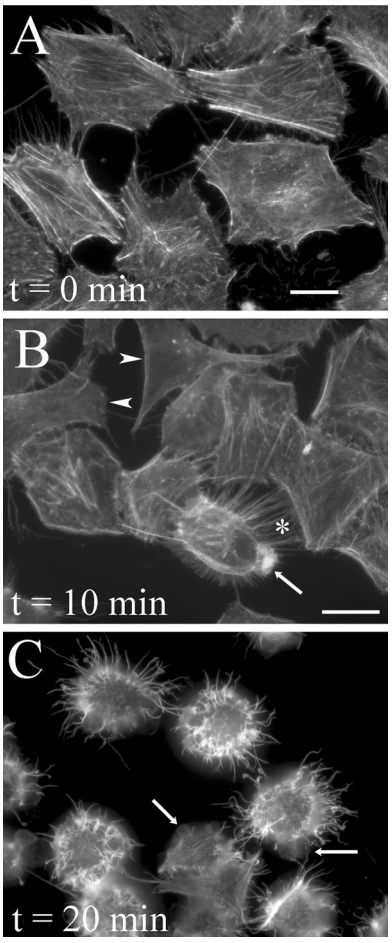

Fig. 5. Panc-1 cells grown on glass coverslips were stained with FITC-phalloidin to observe the actin microfilament network. Cells were untreated (A), or exposed to 50 nM lasonolide A for 10 min (B) or 20 min (C). Arrow heads in B indicate cells with reduced or absent actin stress fibers and microfilaments, and the asterisk marks filopodial extensions. Some bleb structures (B; arrow) were filled with actin, whereas others (C; arrow) had limited actin content. Bars = 10 μm. Studies with actin staining were repeated three times with similar results.

used as controls. Actin readily sedimented into the pellet fraction of the preparation when incubated in buffer F, and when incubated with 20 and 40 μM lasonolide A (Fig. 6), indicating the polymerized state of the protein. Actin remained unpolymerized in the supernatant fraction when kabiramide C was added under polymerizing conditions.

The in vitro effects of lasonolide A on microtubule stability were observed by monitoring the changes in optical density of solutions containing purified tubulin without microtubule-associated proteins. Under these assay conditions, the assembly and disassembly characteristics of tubulin were not altered by the addition of 10 μM lasonolide A.

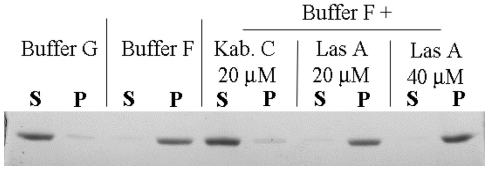

Fig. 6. Purified rabbit smooth actin was incubated in nonpolymerizing (buffer G) or polymerizing (buffer F) buffers and in the presence of kabiramide C (Kab C) or lasonolide A (Las A). After the incubation, samples were centrifuged to pellet any actin polymers. Aliquots of the supernatant (S) and pellet (P) were then separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue to detect the actin protein. This figure is representative of data found in three experiments.

Effect on Membrane Integrity and Cell Recovery. Because of the rapid morphological changes induced by lasonolide A, its effects on membrane integrity were measured in Panc-1 cells 30 and 60 min after exposure (Fig. 7A). Membrane integrity was assessed through the monitoring of LDH activity released into the culture media. At a concentration of 500 nM, lasonolide A did not significantly increase LDH activity compared with cells exposed to media alone. In contrast, Triton X-100, a detergent, induced a large increase in secreted LDH. The presence of lasonolide A in Triton-treated cells did not decrease LDH levels indicating its noninterference with enzyme activity.

A wash-out study was conducted to determine whether the morphological effects of lasonolide A were reversible, or whether the loss of adherence necessarily signaled downstream cell death. Lasonolide A caused a time-dependent reduction in the recovery of Panc-1 cells (Fig. 7B). Almost 80% of Panc-1 cells made nonadherent by 2-h exposure to 50 nM lasonolide A re-attached to the culture plastic and continued proliferating after removal of the compound, whereas

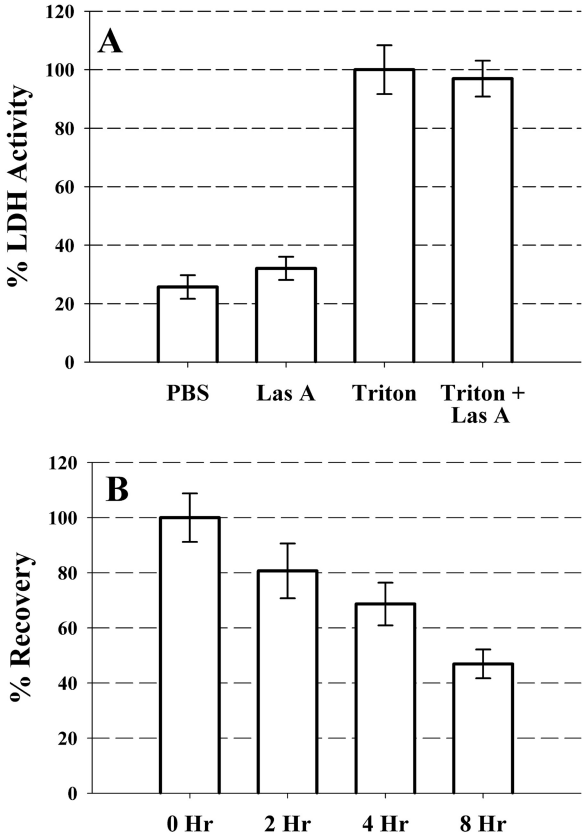

Fig. 7. Membrane integrity and recovery of Panc-1 cells exposed to lasonolide A. A, cells were incubated with 500 nM lasonolide A and/or Triton X-100 for 1 h before measurement of LDH activity released into the media. Exposure to Triton, but not lasonolide A, significantly increased LDH compared with untreated cells (*, $p < 0.05$; data averaged from three experiments). Lasonolide A did not inhibit released LDH activity. B, the majority of Panc-1 cells made nonadherent by 2- and 4-h incubations with 50 nM lasonolide A remained viable when cells were washed and allowed to recover; however, there was a time-dependent significant decrease in the percentage of cells recovered during each of these time points (*, $p < 0.05$). This experiment was repeated three times with similar results.

only 50% of the cells recovered after 8-h exposure. There were no obvious morphological differences to the Panc-1 cells 24 h after their recovery, and they remained sensitive to any further lasonolide A treatment.

Discussion

Lasonolide A was first identified in 1994 with use of a bioassay-guided purification approach in the search for novel antitumor compounds derived from marine organisms (Horton et al., 1994). This polyketide-derived macrolide was isolated from the crude extract of a marine sponge, *Forcepia* sp., and was shown to inhibit the adhesion of PMA-stimulated EL-4.IL-2 cells—an activity intended to correlate with the inhibition of PKC (Osada et al., 1988; Longley and Harmody, 1991). The original assay was based on blebbing induction in human K562 myeloid leukemia cells by PKC-activating compounds (Osada et al., 1988). Although the adhesion inhibitory activity of lasonolide A occurred without apparent immediate damage to the cells, it proved to be highly potent against a wide range of cancer cell lines in cytotoxicity assays after exposure for several days. Our initial studies had suggested that this cytotoxic activity was preferential toward cells of pancreatic origin compared with other cell lines, and this further strengthened the research interest in lasonolide A. However, additional cytotoxicity results and comparison with the growth inhibitory data conducted in the NCI 60 cell panel screen showed that cell lines of pancreatic origin were as highly susceptible to the effects of lasonolide A as many other cancerous cell lines were. Although the data from this multicell line assay can predict a mechanism of action (Paull et al., 1989; Shoemaker, 2006), the results for lasonolide A were not the same as for other known anticancer agents.

Despite the interest in the unique chemical structure and biological activity of lasonolide A, initial research into its pharmacology was delayed because of limited supplies of the natural compound and the difficulty in its synthesis. Later explorations in the U.S. Gulf of Mexico in 2003 yielded sufficient samples of *Forcepia* sp. to resume studies into the biological effects of lasonolide A and helped identify naturally occurring lasonolide analogs (Wright et al., 2004). We confirm here the original findings of lasonolide A cytotoxicity by use of the human Panc-1 and MCF-7 cell lines. The NCI-ADR cell line, which expresses the P-glycoprotein (P-gp) efflux protein, was similarly sensitive to lasonolide A cytotoxicity over Panc-1 and MCF-7 cells, which both lack P-gp. Verapamil, a Pgp inhibitor, did not significantly alter the cytotoxicity of lasonolide A, a further indication that it is not a substrate for P-gp efflux.

Microscopy observations revealed an unusual and rapid morphological response of Panc-1 cells to lasonolide A. Although the contraction and bleb formations were originally assumed to indicate a process of necrosis or apoptosis, the absence of LDH leakage, and the recovery of cells in wash-out studies, demonstrate that these morphological changes are early cellular responses to lasonolide A with the initiation of cell death occurring several hours after a continual loss of adherence.

The speed at which cell contraction and blebbing occurred in response to lasonolide A suggested the activation of the actinomyosin motor complex. Reports of other actin-interactive compounds have shown these agents can dramatically alter cell shape with some similarity to the effects of lasonolide A (Spector et al., 1999). Because of the number of marine-derived natural products that have been demonstrated to affect actin function (Allingham et al., 2006), we explored the possibility of lasonolide A interaction with actin, and its activation of the actinomyosin machinery. Inhibition of myosin II activity with blebbistatin exhibited only a limited ability to reduce the contractile effects of lasonolide A. Although blebbistatin was tested at a concentration 1000 times greater than lasonolide, and approximately 25 times greater than its IC_{50} for myosin IIA and IIB (Straight et al., 2003; Kovács et al., 2004), research by Limouze et al. (2004) indicate that blebbistatin-inhibited myosin retains a weak interaction with actin and does not interfere with the movement of any uninhibited myosin. Therefore, any active myosin remains functional in the presence of neighboring inhibited myosin units. Because the morphological effects of lasonolide A in the presence of blebbistatin were monitored for up to 60 min, it is likely that the resulting contraction and blebbing was due to the cumulative activity of those myosin units that remained active or to their activity as they became temporarily liberated from the reversible inhibition by blebbistatin. In vitro assays with purified actin clearly demonstrate that lasonolide A does not affect actin polymerization or stability, so the observed reduction in stress fiber content after lasonolide A exposure must be an indirect response. It is also unlikely that the reduction in stress fibers is linked to the contraction and blebbing response, because the destabilization of actin fibers with kabiramide C (Tanaka et al., 2003) does not cause morphological effects similar to those observed with lasonolide A.

The initial report from this lab indicated that lasonolide A suppressed PKC activity (Horton et al., 1994), and we investigated this mechanism further. In contrast to this first study we found that lasonolide A is actually a potent PKC activator. However, our results indicate that PKC activation most likely occurs subsequent to the changes in cell shape, leaving the possibility that its activation is more in response to the storm of transduction signals that would surely follow such a dramatic change in cellular architecture, rather than as a near-downstream response to any lasonolide A receptor. This is apparent from the slower onset of PKC phosphorylation (greater at 30 than at 10 min), than that of either p38 or ERK1/2 phosphorylation, or of the morphological changes. In addition, inhibition of PKC activity with BIM-1 abrogated only the phosphorylation of MARCKS, yet had no effect on bleb formation or cell contraction. The activation of PKC is linked through several processes to the loss of stress fibers and the rearrangement of the actin matrix (Larsson, 2006; Rozengurt, 2007). These effects are mediated through multiple substrates of PKC, including MARCKS. Although this finding helps to explain the loss of stress fibers observed in lasonolide A-treated cells, the ineffectiveness of BIM-1 to inhibit the morphological changes suggests that a non-PKC-dependent pathway activates the contraction and blebbing response. The observations that PKC phosphorylation occurs later than those of ERK 1/2 or p38 and that inhibition of PKC activity with BIM-1 had no effect on these MAP kinases indicate that these pathways are not in the same linear cascade induced by lasonolide A. Signal transduction through MAP kinases can occur through a number of external stress-related events and cell receptors, including recep-

tor tyrosine kinases (see Krishna and Narang, 2008, for a review on MAP kinase signaling). To determine whether ERK 1/2 and p38 MAP kinases were activated as downstream signals originating from receptor tyrosine kinases, Panc-1 cells were pretreated with the inhibitor wortmannin before the addition of lasonolide A. To ensure the efficacy of wortmannin at inhibiting receptor tyrosine kinases, Akt phosphorylation was monitored as an immediate-early downstream indicator of the PI3K signal pathway (Takeda et al., 2004). Although Akt was activated in response to lasonolide A, and this effect was reduced by wortmannin, this pretreatment did not inhibit the phosphorylation of either MAP kinase protein or PKC. Together, these data suggest that multiple, parallel signal transduction pathways are initiated by lasonolide A, and include at least PI3K-Akt, PKC-MARCKS, and the p38 and ERK1/2 pathways. However, these data provide few indications as to the originating signal or a possible lasonolide receptor.

The rapid morphological effects of lasonolide A are very similar to those reported to occur in cellular collapse induced by semaphorin signaling through the plexin receptor (Barberis et al., 2004), and are similar to those effects caused by the protein Ser/Thr phosphatase inhibitor calyculin A (Hosoya et al., 1993; Leung et al., 2002). Although these represent two very different mechanisms of activity, both share signal cascade events similar to lasonolide A. These reports provide intriguing clues based on which we can pursue further investigations into the possible mechanisms of lasonolide A activity.

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